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Proliferation of human hepatocellular carcinoma cells from surgically resected specimens under conditionally reprogrammed culture

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Abstract. Hepatocellular carcinoma (HCC) is the third most common cause of cancer mortality worldwide, which is partially due to the lack of appropriate therapeutic options. The development of HCC is accompanied with unique and continuous genomic and epigenetic modifications. Therefore, the absence of a personalized and reproducible human model reduces the ability to determine the potential of candidate treatments. Conditional reprogramming (CR) culture has been used to establish and indefinitely grow patient-derived tumor cell lines in a rapid and efficient manner. In the present study, primary HCC cells were isolated from tumor specimens and cultured under CR conditions. The proliferative potential and capacity of cells to undergo continuous regeneration were evaluated by cell viability and proliferation assays, and the expression of tumor-specific markers was determined by western blotting and immunofluorescence to determine the prospects for use in clinical settings. It was demonstrated that ~55% of tumor samples were able to generate HCC cells that could be continuously expanded and passaged under CR conditions; this ability was associated with the source and composition of the tumor tissues. Furthermore, the expression of the tumor-specific marker α-fetoprotein and the proliferative ability of cells were maintained following cycles of cryopreservation and resuscitation. In conclusion, with further optimization, the CR system may be a useful tool for the precise therapeutic treatment of patients with HCC.

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Abbreviations: HCC, hepatocellular carcinoma; CR, conditional reprogramming; DMEM/F12, Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 medium; AFP, α-fetoproteins

Key words: HCC, conditional reprogramming, tumor specimens, precision therapy

Introduction

Primary liver cancer, predominantly comprising hepatocellular carcinoma (HCC), is reported to be the fifth most common cancer globally and the third most common cause of cancer-associated mortality (1). There is an increasing understanding of the molecular mechanisms that induce hepatocarcinogenesis, including chronic infections such as hepatitis B or C (HBV/HCV), alcohol abuse and metabolic syndromes (2). Surgical resection followed by adjuvant drug therapy is the most common treatment in clinical settings (3); however, the metastasis and chemoresistance of tumor cells results in poor outcomes for patients with advanced HCC (4). As the development of HCC is accompanied with unique and continuous genomic and epigenetic alterations, combining personalized approaches, including molecular analysis-guided targeted therapy and immunotherapy may be a potential strategy to improve the treatment of cancer (5,6). Existing preclinical models comprise genetically engineered mouse models and human tumor-derived cell lines; however, a reproducible human model is required to accurately reproduce the important characteristics of tumors *in vivo* and determine the effectiveness of candidate therapeutics. Commercial tumor cell lines have been extensively used in the investigation of therapeutic targets; however, the establishment of *in vitro* models that use tumor cells from individual patients may serve to improve the clinical relevance of *in vitro* studies (3).

Tumor cells have been associated with strong proliferative ability. This property is detrimental for the rapid expansion of cells derived from adult tumor tissues while retaining stable lineage commitment, particularly from liver tumors (7). Conditional reprogramming (CR) systems have previously been used to establish patient-derived cell lines from normal and tumor tissues that possess the ability to grow indefinitely *in vitro* without genetic manipulation (8,9).

Potential applications for the CR system in clinical settings have been investigated for breast (10,11), lung (12) and prostate cancers (13,14); however, it has been hypothesized that the CR system cannot be used to expand patient-derived metastatic lung cancer cells (15). In an *in vitro* study of cultured liver cancer cells, Broutier *et al* (16) successfully constructed a primary HCC organoid based on the CR system using a three-dimensional (3D) culture method. On the contrary, whether CR may serve as a reliable *in vitro* culture method to obtain matched tumor cells from patients with HCC remains unclear.

The aim of the present study was to establish a culture system with potential clinical applications that enabled the amplification of genetically stable cells. Primary tumor cells were isolated from tissue specimens from 20 patients with HCC and were cultured using the CR system. The proliferative potential and capacity of cells to undergo continuous regeneration, and the expression of tumor-specific markers were evaluated to determine the prospects for use in clinical settings. The study provided a primary investigation into culture systems for HCC cells *in vitro*, in preparation for future studies involving the establishment of a conditionally reprogrammed culture model for drug screening in the treatment of liver cancer drug screening.

Materials and methods

Cell isolation. A total of 20 samples of liver tumor tissue were obtained from patients (10 males and 10 females, aged 38-67 years old) undergoing orthotopic liver transplantation (OLT) or hepatectomy at Tianjin First Center Hospital (Tianjin, China) between January 2015 and December 2017. Tumors were graded using the American Joint Commission on Cancer 8th edition staging system for patients with HCC. The study was approved by the Tianjin First Central Hospital Clinical Research Ethics Committee (review no. 2016N057KY). Informed written consent was obtained from all patients. Procedures were conducted in accordance with the Declaration of Helsinki (17). Surgically resected liver tumor tissue was obtained from individuals with HCC who had no history of viral-mediated hepatitis. Half of the tissue was de-identified and supplied to lab personnel for tissue culture; remaining tissue was used for histological analysis. A 1 cm^3 section of tumor tissue was collected and transferred into a sterile tube containing 1x cell protective fluid (Beijing Percans Oncology Research Co., Ltd., Beijing, China). The tissues were qualitatively divided into two types: Hard tissues (brittle and non-viscous tissues) and soft tissues (tissues with loose texture consisting of lumpy masses). Following five washes with PBS, the samples were minced into ~2 mm^3 sections and treated using a gentleMACS™ Tissue Dissociator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The samples were digested into single cells by 1x collagenase/hyaluronidase solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) followed by 0.25% trypsin-EDTA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 2 h. Cell suspensions were filtered using a MACS® SmartStrainer (Miltenyi Biotec GmbH). The primary cells were suspended in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 medium (DMEM/F12; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and then centrifuged at 300 x g for 5 min at 37˚C. Primary HCC cells were further purified from stromal cells using a magnetic bead isolate system (Beijing Percans Oncology Research Co., Ltd.) according to the manufacturer's protocols (18).

H&E staining. The tumor tissues were fixed with 4% paraformaldehyde overnight at 4˚C and embedded in paraffin. The samples in paraffin were cut into 3-μm sections, dewaxed in xylene and rehydrated in a graded alcohol series. Sections were stained with hematoxylin for 10 min at room temperature, and rinsed with tap water for 10 min. Differentiation was performed with differentiation buffer in the H&E staining kit (Beyotime Institute of Technology, Shanghai, China) for 30 sec at room temperature to the obtain clear structure. Following further washing in running tap water for 10 min, the sections were stained with eosin for 2 min at room temperature, then dehydrated and mounted. Then, sections were imaged using an optical microscope (magnification, x40; Eclipse 80i; Nikon Corporation, Tokyo, Japan); three images/sample were acquired for analysis.

CR culture. In the CR system, mouse embryonic fibroblast cells (NIH-3T3; China Center for Type Culture Collection, Wuhan, China) were irradiated at 30 Gy with gamma radiation to provide an irradiated fibroblast feeder layer. HCC cells were passaged in DMEM/F12 containing the following: 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.); 10 μg/ml Y-27632 (Enzo Life Sciences, Inc., Farmingdale, NY, USA); 0.4 μg/ml hydrocortisone (Sigma-Aldrich; Merck KGaA); 5 μg/ml insulin (Thermo Fisher Scientific, Inc.); 8.6 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA); 20 ng/ml epidermal growth factor (Sigma-Aldrich; Merck KGaA), 20 ng/ml hepatocyte growth factor (Sigma-Aldrich; Merck KGaA), 24 μg/ml adenine (Sigma-Aldrich; Merck KGaA) and 3 nM dexamethasone (Sigma-Aldrich; Merck KGaA). The feeder cells were removed following incubation for 1 min in 0.05% Trypsin-EDTA (Sigma-Aldrich; Merck KGaA) at 37˚C and replaced with fresh cells ($4x10^4$ cells/cm^2) during the passage of HCC-CR cells. A 6-day culture period was required to generate larger cell clones prior to the initial passage of P0 cells, then the cells were passaged every 3-4 days. The HCC-CR cells were sub-cultured at a 1:3 ratio upon reaching 60-80% confluence, and these cells were cultured for a total of 6 generations in a 5% CO_2 incubator at 37˚C. Subsequent passages of the HCC-CR cells were referred to in a sequential numerical order starting at P1. HCC-CR cells were observed using an optical microscope (magnification, x10; Olympus Corporation, Tokyo, Japan) following culture for 50 and 100 h.

Cell colony formation and viability assay. Following culturing under CR conditions for six passages, the cell colony formation per passage was observed using an inverted phase-contrast microscope (magnification, x10; Olympus Corporation). The HCC-CR cells were stained using a Live/Dead Molecular Probes staining kit (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37˚C and then analyzed using an inverted fluorescence microscope (magnification, x10; Olympus Corporation). Three images/sample were acquired for analysis.

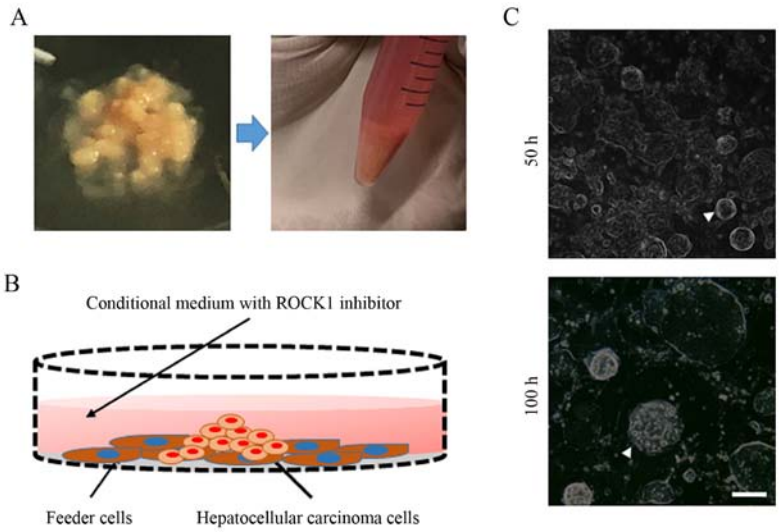

Figure 1. Isolation and CR culture of primary HCC cells. (A) Acquisition of HCC cells from tumor specimens. (B) Schematic diagram of CR culture. (C) HCC cell colonies following CR culture. Scale bar=100 μm. CR, conditional reprogramming; HCC, hepatocellular carcinoma; ROCK1, rho-associated coiled-coil containing protein kinase 1.

Cell proliferation assay. The proliferative ability of the P0 HCC-CR cells following culturing for 10 days was quantified via 5-ethynyl-2'-deoxyuridine (EdU)/Hoechst 33342 staining using a Cell-Light™ EdU *in vitro* imaging kit (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Briefly, HCC-CR cells ($4x10^4$ cells/cm^2) were seeded into a 24-well plate and incubated with 50 mM EdU labeling solution (200 ml) at 37˚C under 5% CO_2 for 3 h. The HCC-CR cells were then sequentially treated with 4% paraformaldehyde (PFA; pH 7.4) for 30 min, 2 mg/ml glycine for 5 min, 0.5% Triton X-100 for 10 min, anti-EdU working solution for 30 min and 5 mg/ml Hoechst 33342 dye for 30 min (all at room temperature). The cells were imaged under a fluorescence microscope (magnification, x10; Leica Microsystems GmbH, Wetzlar, Germany). Three images/sample were acquired for analysis. The numbers of HCC-CR cells were counted for each passage, and a plot of accumulated population doublings versus growth days was constructed following culturing for 10, 14, 22 and 30 days as previously described (19).

Western blotting. HCC-CR cells were separated from feeder cells by differential trypsinization. Briefly, the cells were washed by PBS, and then incubated by 0.05% trypsinization for 1 min at 37˚C under 5% CO_2. The feeder cells were separated by tapping the bottom of the plates. Then, total protein was extracted from HCC-CR cells using lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA and protease inhibitor cocktail, pH 7.4) on ice. The lysates were centrifuged at 14,000 x g for 10 min at 4˚C. The supernatants were then collected and the concentration of total protein was determined using a bicinchoninic acid assay kit (Beyotime Institute of Technology) according to the manufacturer's protocols. Equal amount of protein (30 μg/lane) of the samples were boiled in water with SDS-PAGE sample loading buffer (Beyotime Institute of Technology) for 10 min prior to separation via 10% SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (Roche Diagnostics, Basel, Switzerland) and blocked with 5% non-fat milk at room temperature for 1 h. Then the proteins. Then the membranes were incubated with primary antibodies against α-fetoprotein (AFP; 1:1,000; cat. no. 4448; Cell Signaling Technology, Inc., Danvers, USA) and β-actin (1:1,000; cat. no. AF0003; Beyotime Institute of Technology). The membranes were then incubated with horseradish peroxide-conjugated secondary antibodies (1:1,000; cat. nos. A0208 and A0216; Beyotime Institute of Technology) for 1 h at room temperature and washed with TBS containing 1% Tween and PBS. Protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Pierce; Thermo Fisher Scientific, Inc.) with an ECL kit (EMD Millipore, Billerica, MA, USA). Protein expression was quantified using Quantity One 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunofluorescence staining. HCC-CR cells ($4x10^4$ cells/cm^2) were seeded into a 24-well plate and incubated with 50 mM EdU labeling solution (200 ml) at 37˚C under 5% CO_2 for 3 h. Then the cells were fixed with 4% (v/v) PFA for 10 min at room temperature, permeabilized with 0.3% Triton X-100 and then blocked with 1% (w/v) bovine serum albumin (cat. no. P0023B; Beyotime Institute of Technology) at 37˚C for 30 min. Following incubation overnight at 4˚C with a primary antibody (mouse anti-human AFP; 1:200; cat. no. ab3980; Abcam, Cambridge, UK), the cells were washed with PBS and incubated with an Alexa Fluor® 488-conjugated secondary antibody (1:1,000; cat. no. R37120; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The cells were then incubated with anti-EdU working solution for 30 min and 100 ng/ml DAPI (Beyotime Institute of Technology, Shanghai, China) for 5 min (all at room temperature). and the samples were analyzed using a confocal laser scanning microscope (magnification, x10; SP5; Leica Microsystems GmbH, Wetzlar, Germany).

Statistical analysis. Data were analyzed using using SPSS software 22.0 (SPSS, Inc., Chicago, IL, USA). AFP protein expression data were analyzed by one-way analysis of variance, whereas cell proliferation data at different time points

Table I. Clinicopathological data of patients.

Subject No.	Age	Sex	Grading	Metastasis	Differentiation	Surgical method	Necrosis (%)	Fibrosis (%)	CR culture passage no.
1	46	Female	T2	N1M1	III	OLT	0	10	P6
2	59	Male	T3	N1M0	II	Hepatectomy	10	0	P6
3	52	Female	T1	N0M0	I	OLT	30	30	P2
4	47	Male	T2	N0M0	II	OLT	10	10	P6
5	53	Female	T3	N1M1	III	Hepatectomy	10	30	P6
6	44	Female	T2	N0M0	I	OLT	10	15	P6
7	58	Male	T1	N0M0	I	OLT	20	30	P2
8	54	Male	T3	N0M0	II	Hepatectomy	15	25	P4
9	62	Female	T2	N0M0	II	OLT	20	25	P3
10	41	Male	T2	N1M0	III	OLT	0	5	P6
11	59	Female	T3	N1M0	III	Hepatectomy	0	15	P6
12	67	Male	T2	N0M0	II	OLT	60	0	N/A
13	43	Female	T2	N0M0	II	OLT	20	0	P6
14	53	Male	T2	N0M0	II	OLT	15	5	P6
15	65	Male	T1	N0M0	II	OLT	30	35	N/A
16	62	Female	T2	N2M1	III	OLT	20	0	P6
17	51	Female	T3	N0M0	II	Hepatectomy	60	0	N/A
18	38	Female	T4	N1M1	III	Hepatectomy	10	5	P6
19	61	Male	T2	N0M0	II	OLT	30	15	P4
20	51	Male	T3	N0M0	II	Hepatectomy	20	5	P6

CR, conditioned reprogramming; P2-6, cells exhibited the ability to passage to P2-6; N/A, not applicable, OLT; orthotopic liver transplantation.

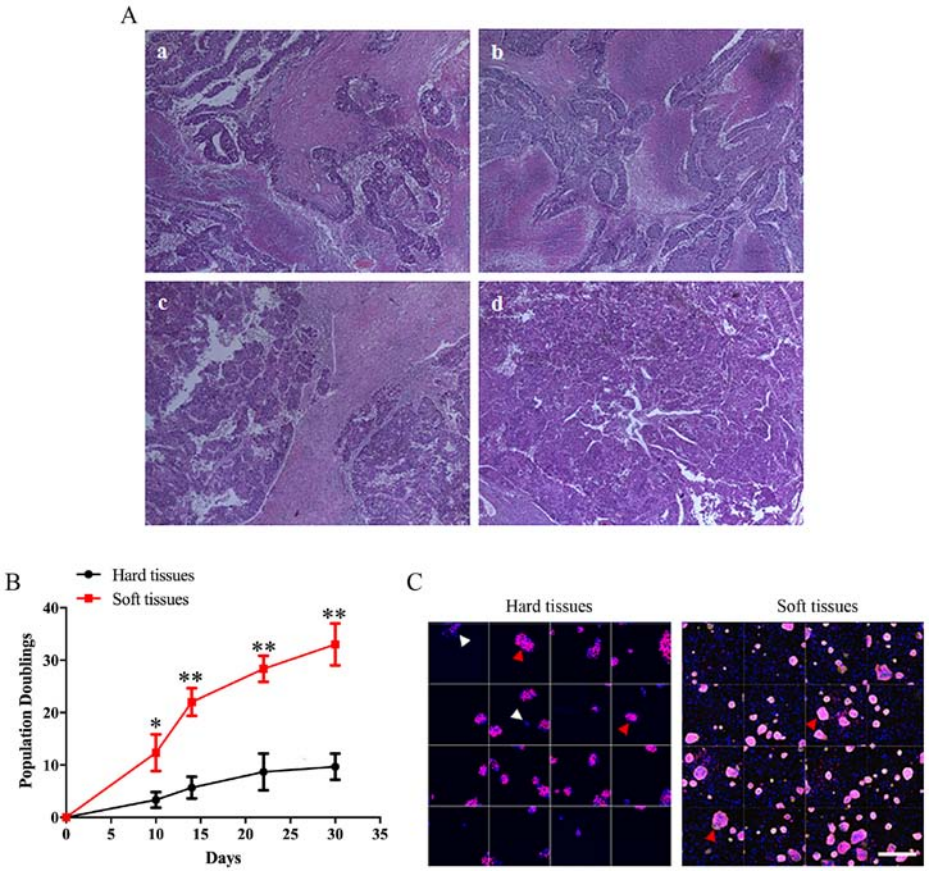

Figure 2. Proliferative ability of HCC-CR cells. (A) H&E staining of tumor tissues (magnification, x40). A-a, II-differentiated tissue with 30% necrosis and 30% fibrosis; A-b, II-differentiated tissue with 60% necrosis; A-c, III-differentiated tissue with 35% fibrosis and A-d, III-differentiated tissue with no necrosis and fibrosis. (B) Population doublings of HCC-CR cells isolated from hard and soft tissues were detected following culturing for 10, 14, 22 and 30 days. (C) 5-Ethynyl-2'-deoxyuridine (red) and Hoechst 33342 (blue) staining of HCC-CR cells following culture for 10 days. A notable increase in the number of colonies were observed from cells of soft tissue compared with hard tissue (white arrows); fewer proliferative cells were observed in hard tissues compared with soft tissues (red arrows). Scale bar=300 μm. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 vs. hard tissues. HCC-CR, conditioned reprogramming culture of hepatocellular carcinoma cells.

were analyzed using Student's t-tests. Data were presented as the mean ± standard deviation of at least three independent experiments. Associations between the success rate of HCC-CR culture and patients' background, including gender and surgical method, were analyzed with χ^2 tests. P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of patient-derived primary HCC-CR cell cultures. Tumor tissue samples from 20 patients with HCC that had not been previously treated with radiotherapy or chemotherapy were collected, harvested and used for cell culture in the present study (Fig. 1A). Cell cultures were established by seeding HCC cells onto an NIH-3T3 fibroblast feeder layer in CR medium (Fig. 1B). As presented in Fig. 1C, adherent HCC cells exhibited small colonies at 50 h and progressed into cell islands by 100 h.

The clinicopathological data of the patients are presented in Table I. The median age of the patients was 54.7 years; all patients suffered from HBV and/or HCV. Tissue samples were obtained during OLT or hepatectomy. A total of 35% (7/20) of patients exhibited T3/T4 grade tumors. Additionally, 35% (7/20) of patients had tumor metastasis. HCC cells from 55% of tumor tissues underwent successful continuous passaging under CR conditions; the ability to establish continuous passaging was markedly associated with the source and composition of tumor tissues. With the exception of tissues from 100% of T1 patients and 54.5% of T2 patients, HCC cells were obtained via CR culture from tissues from all other patients. HCC cells from metastatic patients were more likely to be expanded *in vitro*. The success rate of establishing CR cultures was independent of the surgical method used (P=0.9510). HCC-CR cells were not successfully generated from tissues with a necrosis rate >30% and a stromal ratio >35%.

Propagation potential of primary HCC-CR cells. Complete hepatic nodule or blood vessel-rich tissues in primary HCC lesions were selected for further CR culture. A 6-day culture period was required to generate larger cell clones prior to the initial passage of P0 cells, after which HCC cells were generated every 3-4 days. CR cultures were determined to possess the potential for continuous generation providing cells were generated on a 3-4-day cycle, and colonies demonstrated a continuous increase in quantity and volume. The morphologies of various tumor tissues were observed by H&E staining (Fig. 2A). Harder tissues exhibited larger fibrotic areas and smaller necrotic regions. As presented in Fig. 2B, the

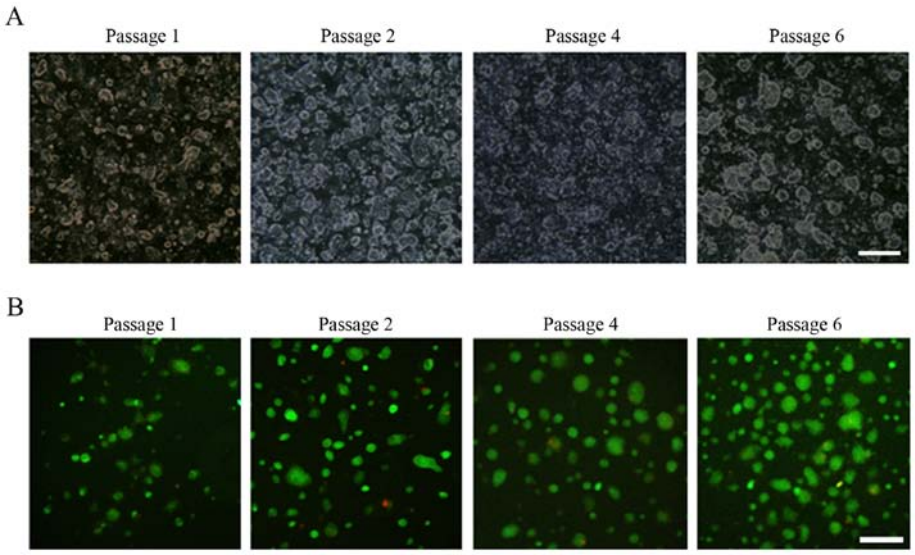

Figure 3. Continuous generation capacity of HCC-CR cells. (A) Cell colony formation per passage was observed using an inverted phase-contrast microscope. (B) Live (green) and dead (red) staining of HCC-CR cell colonies during Passage 1, 2, 4 and 6. Scale bar=100 μm. HCC-CR, conditioned reprogramming culture of hepatocellular carcinoma cells.

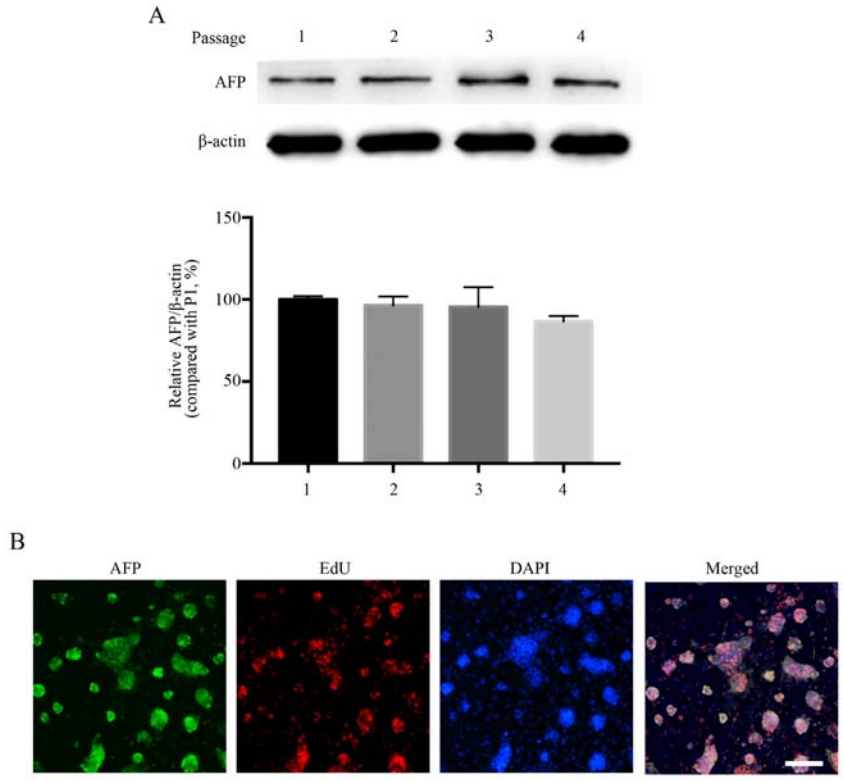

Figure 4. Tumor-specific marker expression of HCC-CR cells. (A) Expression of AFP in HCC-CR cells as determined via western blotting. (B) Staining of HCC-CR cells cultured for 40 days. AFP was stained by Alexa Fluor® 488 (green), proliferative nuclei were stained by EdU (red) and nuclei were stained by DAPI (blue). Scale bar=100 μm. AFP, α-fetoprotein; EdU, 5-ethynyl-2'-deoxyuridine; HCC-CR, conditioned reprogramming culture of hepatocellular carcinoma cells.

proliferation of cells obtained from brittle, non-viscous tissues (hard tissues) during continuous cultivation was significantly decreased compared with cells from soft tissues with lumpy masses. Additionally, following culture for 6 days, a markedly increased number of colonies was observed in the soft tissues group compared with in the hard tissues group (Fig. 2C). Furthermore, EdU staining revealed that the number of cells with proliferative potential in hard tissues cell colonies was notably reduced compared with in soft tissue cell colonies.

Continuous generation capacity of HCC-CR cells. HCC-CR cells that possessed proliferative ability in the CR system were subjected to cryopreservation and resuscitation during every passage. As presented in Fig. 3A, HCC-CR cell colonies

exhibited continuous increases in quantity and volume across generations. Additionally, live/dead cell staining revealed that HCC-CR cells retained high viability following repeated cryopreservation and resuscitation (Fig. 3B).

Expression of AFP in HCC-CR cells. The identity of the cultured cells was demonstrated by the stable expression of the HCC marker AFP during continuous generation (Fig. 4A). AFP expression was markedly downregulated with increasing passages; however, there was no significant difference. Immunofluorescence and EdU staining revealed that the cell purity was >90% and the majority of AFP-positive cells possessed high proliferative potential (Fig. 4B).

Discussion

It was previously hypothesized that only a small proportion of tumor cells were able to form colonies *in vitro* (20). In particular, certain human epithelial cells, including those from the prostate, lung and liver, possess very short lifespans *in vitro* and can only undergo a limited number of passages, reducing their potential use in cell biology studies (9). A novel culture system has been developed for the indefinite propagation of various primary human cells (normal and tumor cells) *in vitro* via co-culture with irradiated fibroblast feeder cells and the rho-associated coiled-coil containing protein kinase 1 inhibitor Y-27632 (21). This culture system is termed CR due to the conditional induction of cell proliferation. As self-renewal is a reported property of stem cells, it was hypothesized that CR culture induced adult cells to exhibit adult stem cell-associated properties (22).

The aim of the present study was to establish a culture system *in vitro* for human hepatoma cells. Recent studies have reported the ability of cancer cells to exhibit sustained stable amplification under CR conditions (10-14). Numerous studies have made advancements in the field of liver cancer cell amplification; however, sufficient rapid amplification of cells was not established for clinical use (23). A previous study reported the successful construction of a primary HCC organoid; however, the expansion time required to generate the 3D organoid was impractically long (16). In the present study, HCC cells were passaged every 3-4 days. The numbers of harvested cells were counted following each passage, and a constant growth of HCC-CR cells during each passage was observed for 30 days (Fig. 2B). These findings were consistent with previous studies and meet clinical requirements (14,24). The identification of additional biomarkers is required to further verify the reliability of the *in vitro* CR culture method in obtaining matched tumor cells from tissues from patients with HCC.

The ability to expand cells isolated from tissue samples *in vitro* was associated with the quality of the original tissue. In the present study, the HCC cells that were continuously passaged under CR conditions were obtained from ~55% of tumor samples; this ability was affected by the origin and volume of actively proliferating tissue, as HCC cells from necrotic or fibrotic tissues struggled to be continuously passaged. As presented in Table I, the success rate of HCC-CR culture was independent of the age of patients and the surgical method used, which was consistent with a study investigating nasopharyngeal carcinoma (25). Additionally, the proliferation and cloning abilities of cells were notably increased in tissue samples from patients with metastasis compared with in other samples. Cells for amplification could not be isolated from calcified and necrotic lesions. Furthermore, reduced proliferation and activation was observed in tumor cells isolated from fibrotic samples. Based upon these findings, to obtain HCC-CR cells that can be used for subsequent experiments, original samples should be extracted from patients with tumors in the T2/T3 stage. Furthermore, combined with the sample information and the cell culture effect, it was suggested that the tumor tissue should be >1 cm^3 in size, with a necrosis rate of <50% and a stromal ratio of <40% to achieve acceptable expansion.

Increases in the number and area of cell colonies are regarded as manifestations of the proliferation of tumor cells *in vitro* (26). In the present study, successfully amplified HCC-CR cells formed more and larger cell colonies, thereby exhibiting adaptability to the CR medium. Furthermore, the proliferation of cells, formation of colonies and expression of AFP were markedly unaffected by repeated cycles of cryopreservation and resuscitation, indicating that the CR system met the requirements for successful hepatoma cell culture. On the contrary, due to the apparent complexity of the HCC-CR medium and the diversity of clinical samples, further investigation is required to develop a simpler, more versatile CR medium. Compared with tumor cells cultured under CR conditions, commercial tumor cell lines fail to fully reproduce important features of tumors *in vivo*. The heterogeneity of tumors results in susceptibility to drug resistance, leading to tumor recurrence and metastasis. In clinical settings, cells cultured under CR conditions may be useful for drug screening. In the present study, tumor cells were divided into soft and hard tissue groups. In addition, stable expression of the HCC marker AFP during continuous passaging was determined via western blotting. AFP is the most extensively studied serological biomarker in the surveillance of HCC and the only marker that has undergone all five phases of biomarker development (27,28). The findings of the present study are promising; however, the CR system varies from physiological conditions. Therefore, analysis at the transcriptional and translation levels should be conducted, and *in vivo* transplants of CR-derived tumors should be performed.

In conclusion, 20 tumor specimens from patients with HCC were collected. HCC cells isolated from 55% of samples exhibited continuous expansion under CR conditions; the ability to undergo continuous passaging was associated with the source and composition of the original tumor tissues. The expression of AFP in HCC-CR cells was stable during passaging, and cells demonstrated adaptability to CR culture conditions. These findings indicated that the CR system may be a useful source for passaging HCC cells required for clinical trials; however, the specific media components require further optimization.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

ZW made substantial contributions to the conception and design of the present study, and the acquisition, analysis and interpretation of data. BB and HS performed the experiments. LL collected and analyzed patient information, and was involved in drafting the manuscript and revising it critically for important intellectual content. HZ performed some of the experiments and provided final approval for the present version to be published. SW was involved in the analysis and interpretation of data. ZS made substantial contributions to the design of the study and agreed to be accountable for all aspects of the study in ensuring that questions regarding the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Tianjin First Central Hospital Clinical Research Ethics Committee (review no. 2016N057KY).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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